Biochemical and Molecular Predictors for Prognosis in Nonketotic Hyperglycinemia

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Abstract:

Objective: Nonketotic hyperglycinemia is a neurometabolic disorder characterized by intellectual disability, seizures, and spasticity. Patients with attenuated nonketotic hyperglycinemia make variable developmental progress. Predictive factors have not been systematically assessed.

Methods: We reviewed 124 patients stratified by developmental outcome for biochemical and molecular predictive factors. Missense mutations were expressed to quantify residual activity using a new assay.

Results: Patients with severe nonketotic hyperglycinemia required multiple anticonvulsants whereas patients with developmental quotient (DQ)>30 did not require anticonvulsants. Brain malformations occurred mainly in patients with severe nonketotic hyperglycinemia (71%) but rarely in patients with attenuated nonketotic hyperglycinemia (7.5%). Neonatal presentation did not correlate with outcome, but age of onset ≥4 months was associated with attenuated nonketotic hyperglycinemia. CSF glycine levels and CSF:plasma glycine ratio correlated inversely with DQ; a CSF glycine > 230 μM indicated severe outcome and CSF:plasma glycine ratio ≤0.08 predicted attenuated outcome. The glycine index correlated strongly with outcome. Molecular analysis identified 99% of mutant alleles including 96 novel mutations. Mutations near the active cleft of the P-protein maintained stable protein levels. Presence of one mutation with residual activity was necessary but not sufficient for attenuated outcome; two such mutations conferred best outcome. Divergent outcomes for the same genotype indicate a contribution of other genetic or nongenetic factors.

Interpretation: Accurate prediction of outcome is possible in most patients. A combination of four factors available neonatally predicted 78% of severe and 49% of attenuated patients, and a score based on mutation severity predicted outcome with 70% sensitivity and 97% specificity.

Introduction

Nonketotic hyperglycinemia (NKH, glycine encephalopathy, MIM 605899) is an autosomal recessive neurometabolic condition caused by deficient activity of the glycine cleavage enzyme system. Patients usually present in the neonatal period with increasing lethargy, coma, seizures, and apnea, which spontaneously resolves after 1 to 3 weeks.[1-3] They develop intellectual disability, seizures, persistent axial hypotonia, and spasticity. Some patients have congenital brain malformations including agenesis of the corpus callosum or a retrocerebellar cyst with hydrocephalus.[4-6] Patients with attenuated NKH exhibit a variable degree of intellectual disability and seizures, hyperactivity, chorea, and intermittent episodes of lethargy and ataxia.[2,3,7,8] Up to 15% of neonatally presenting patients and 50% of infancy presenting patients show this attenuated phenotype,[2,3] but elements predicting the neurodevelopmental outcome in NKH have not been systematically studied.

The glycine cleavage enzyme complex is composed of four proteins. The P-protein, encoded by the *GLDC* gene, is a pyridoxal phosphate-dependent glycine decarboxylase, the H-protein, encoded by the *GCSH* gene, is a lipoic acid containing hydrogen-carrier protein, the T-protein, encoded by the *AMT* gene, is a tetrahydrofolate-dependent protein, and the L-protein is a lipoamide dehydrogenase. Patients with classic NKH have mutations in *GLDC* or *AMT*, including exonic deletions in *GLDC*.[9-11] The glycine cleavage enzyme is expressed in liver, brain and placenta, and at low levels in Epstein-Barr virus transformed lymphoblasts.[12] Deficient enzyme activity causes elevated glycine levels in plasma and CSF with an elevated CSF:plasma glycine ratio.[13] Lower CSF glycine levels and a lower ratio were reported for some patients with attenuated NKH.[7,14] Treatment most commonly involves sodium benzoate given to lower plasma glycine levels, and dextromethorphan or ketamine with the intent to reduce the excessive stimulating activity of glycine on NMDA receptors, with unclear effect on outcome.

Predicting the prognosis of patients with NKH is important for families to make decisions on treatment following diagnosis, particularly in the neonatal intensive care setting where withdrawal of life-sustaining therapy may be considered.[15] Differences in treatment response may exist between outcome categories, requiring early identification for appropriate treatment. In this study, we reviewed the outcome of patients with NKH in relation to biochemical, enzymatic, and molecular markers in addition to basic clinical and radiological findings.

Methods

Clinical studies. The primary study was approved by the ethics committees of the Colorado Multiple Institutional Review Board and Catholic University Leuven, and written informed consent was obtained from all study subjects. A second limited exempt chart review study was approved by the Colorado Multiple Institutional Review Board. Some clinical details of 12 patients were previously described.[2,5,16-18] All patients had classic NKH evidenced by elevated CSF glycine levels, elevated CSF:plasma glycine ratio, normal urine organic acids, and mutations in either *GLDC* or *AMT* excluding variant NKH[19,20]. Medical records were abstracted through a questionnaire with data listed in Supplementary Table, and a clinical investigator (JVH or JH) examined the vast majority of patients. Age at symptom onset was considered neonatal if symptoms occurred in the first week of life, with later onset specified. Glycine levels were recorded at diagnosis, excluding blood contaminated CSF samples.[20] Brain magnetic resonance imaging (MRI) reports were reviewed for structural abnormalities. Since in NKH seizure propensity increases during the first year of life and improves on benzoate treatment, therapy resistance of the seizure disorder was estimated from the number of concurrent antiepileptic drugs (AED) used after age 1 year and while on benzoate therapy.[2] The glycine index was calculated by subtracting the molar glycine intake in food from a dietary recall from the molar dose of sodium benzoate needed to normalize

plasma glycine levels divided by body weight, which reflects a whole body balance of glycine metabolism.[18] Enzyme assay results were recorded. Neurodevelopmental outcome was systematically assessed. Patients whose sole developmental milestone consisted of the ability to smile were given a developmental age of ≤ 6 weeks and categorized as severe NKH. Those making developmental progress beyond this stage were categorized as attenuated NKH, and formal developmental testing at an age > 2 years was used to derive a developmental quotient (DQ). Typical measures used were Bayley Scales of Infant Development, Mullen Scales of Early Learning, or Wechsler Scale of Intelligence. For each child, a developmental quotient (DQ) was calculated. The developmental quotient is a measure of the rate of development. By using the mean or median age at which a milestone presents, a functional age equivalent (AE) for a child's development is given. It is the ratio of development age to chronological age. Subjects were subcategorized as poor attenuated NKH if DQ < 20, as intermediate attenuated NKH if DQ ≥ 20 and < 50, and as mild attenuated NKH if DQ ≥ 50. Patients without formal developmental assessment were categorized as attenuated NKH, not otherwise specified, if they could grasp and sit or walk independently; and as severe NKH if they could only smile.

Molecular studies. Exons and at least 50 nucleotides into the flanking intron sequences of *AMT* (GenBank® NM_000481.2, NP_000472.2, Ensembl ENSG00000145020) and *GLDC* (GenBank® NM_000170.2, NP_000161.2; Ensembl ENSG00000178445) were sequenced. Phase was established from parental samples. Deletions and duplications in *GLDC* were analyzed by either multiplex ligation dependent probe amplification[10], or, by a targeted array-based comparative genomic hybridization utilizing a customized 60k oligonucleotide microarray with a positive threshold of 3 consecutive probes allowing the detection of 300 bp deletions, and verified by dual real-time PCR with Taqman® probes. Sequence variants were compared with six other species and conservation recorded. Splice site mutations were analyzed *in silico* using BDGP Splice Site Prediction by Neural Network program,[21] and

for select patients by analysis of mRNA in EBV transformed lymphoblasts. To evaluate the effect on splicing of a deep intronic mutation in *AMT* (patient 75), the generated cDNA was sequenced in two overlapping fragments. To evaluate if the maternal mutation c.2203-2A>G; IVS18-2A>G (patient 310) created a leaky splicing error, we evaluated for the presence of normally spliced mRNA not containing the paternal mutation c.547delG in the mRNA of the patient and mother by digestion of the cDNA with NcoI followed by agarose electrophoresis, where the paternal mutation abolishes a restriction site.

Protein expression and modeling studies. The impact of each mutation on residual enzyme activity was systematically evaluated. Exonic deletions, nonsense mutations, and frameshift mutations were categorized as having no residual activity, except for a frameshift mutation that affected the final 20 amino acids. Splice site mutations affecting the conserved two donor or acceptor nucleotides and predicted *in silico* as likely affecting splicing, were considered to leave no residual activity.
For missense mutations in *GLDC*, the impact on residual P-protein activity was assessed by expression *in vitro* in COS cells and measuring the residual enzyme activity on a glycine exchange assay modified by the use of purified human recombinant lipoylated H-protein.[22] Missense mutations were introduced by site directed mutagenesis into a full length *GLDC* cDNA in the pCMV6-Entry vector (RC211292, Origine Rockville, MD, USA), and with mock and wild type *GLDC* included in each experiment, were transfected into COS7 cells using lipofectamine 2000 (Life Technologies™, Carlsbad, CA, USA). Cells were harvested after 48 hours and P-protein activity was measured by the glycine exchange assay containing excess lipoylated, recombinant human H-protein.[22] Activities were normalized by protein concentration measured by the Bradford method[23] and mutant enzyme activity expressed as percentage of concurrent wild-type enzyme activity determined in the same experiment. Transfection efficiency was monitored by qPCR for *GLDC* cDNA content. To generate recombinant, lipoylated human H-protein, *GCSH* cDNA PCR amplified from a human liver cDNA library was cloned as a thioredoxin fusion protein into the pBAD-

DEST 49 vector (Life Technologies™, Carlsbad, CA, USA), expressed in bacteria, and purified from the cell lysate by boiling for one minute followed by chromatography on a DEAE cellulose column. Lipoate-protein ligase (*LplA*) from *E. coli* was expressed, purified and used to lipoylate purified human H-protein as described using a molar ratio of 0.5:1.0 LplA:H-protein.[24] Lipoylation was confirmed by western bloting (Abcam, ab97625, Cambridge, MA, USA) and by mass spectrometry. The N-terminal thioredoxin tag was removed from the lipoylated H-protein by enterokinase digestion (New England Biolabs, Ipswich, MA, USA), and reaction products separated using a Q sepharose FastFlow.

Following transfection in COS cells, steady state P-protein levels were identified by Western blotting following SDS-PAGE using an antibody against the P-protein from Abcam (ab97625), with β-actin as a loading control. Co-expression studies used co-transfection with vectors containing both mutants compared to transfection with a single mutation and an empty vector. A model of human P-protein encoded by the *GLDC* gene was created using the homology modeling program ESyPred3D based on the crystal structure of holo glycine decarboxylase from *Synechocystis* (pdb id: 4LHC)[25,26]. The relative locations of mutated residues in the three-dimensional model were viewed using the program RasTop 2.2 (http://www.geneinfinity.org/rastop/).

Mutation score: A mutation score was constructed as the sum of both alleles with a mutation without residual activity scored -1 and with residual activity +2. For the presumed mutation score, the *AMT* mutations p.M1T and p.R320H were considered to have no residual activity and the mutation p.I106T to have residual activity based on the occurrence in multiple families. Thus the presence of two mutations without residual activity will give a score of -2, whereas the presence of at least one mutation with known residual activity will give a score of ≥1.

Statistics. Descriptive statistics were used to summarize the data. The difference between severe and attenuated variables is evaluated by student t-test for variables that have normal distribution, and by the Mann-Whitney-U test (MWU) for variables that are not normally distributed as evaluated with Wilk-Shapiro. Subcategories of attenuated NKH were evaluated by one-way analysis of variance. The significance of a difference in proportion is calculated by the Chi-square test. Pearson's and Spearman's correlation tests were conducted to identify the correlation between outcomes and predictors, and identify colinearity among the predictors according to the data distribution. Multiple linear regression was performed to model the developmental quotient in relation to predictors. Multiple ordinal logistic regression was performed to model the relationship between predictors and disease severity or antiepileptic drugs. IBM SPSS Statistics package version 21 (IBM, Armonk, NY) and SAS Version 9.3 (SAS, Cary, NC) were used to perform the analyses. A p value < 0.05 was set to consider statistical significance.

Results

Clinical severity: Developmental Outcome, Seizures, and Brain Malformations. Of 124 patients, there were 26 patients (21%) who died in the neonatal or early infantile period. The 56 patients (45%) with severe outcome NKH represent a uniform very poor development limited to smiling, or rarely able to roll from side to prone. There were 42 patients (34%) with attenuated NKH: 6 patients with poor attenuated NKH (DQ< 20), 12 intermediate attenuated NKH (DQ20-50), 15 mild attenuated NKH (DQ≥50), and 9 attenuated NKH where the DQ was not available. The development presented a continuum from a DQ <10 up to the normal range with IQ of 82 in a child who follows regular education curriculum with assistance. The number of antiepileptic drugs used for seizure control related to the degree of developmental delay. All patients with severe NKH were treated with multiple (≥2) anticonvulsants and had persistent seizures, whereas patients with a DQ >30 never required anticonvulsant therapy. The difference between severe NKH patients (mean 3.1 AED), attenuated poor and intermediate NKH

combined (mean 1 AED), and mild NKH (mean 0 AED) was significant ($p<0.001$) (Fig 1A). In patients with severe NKH, 71% had brain malformations identified on MRI, whereas only 7.5% of patients with attenuated NKH had malformations, all hypoplastic corpus callosum (Chi-square $p<0.001$) (Table 1). Severe brain malformations (corpus callosum agenesis or cerebellar cyst with hydrocephalus) only occurred in patients with severe NKH or in neonatal death. Thus, three features related to clinical severity, developmental delay, number of AED, and brain malformation, interrelated as a class. We next examined clinical predictors of these severity classes.

Basic Predictors: Age of onset and gender: Age at onset was commonly neonatal in all categories (Table 1). Onset delayed beyond the first week was present in 12% of patients with severe NKH, and in 52% of patients with attenuated NKH. Delayed onset in severe NKH was between 2 weeks and 2 months, and ≤ 4 months of age in poor or intermediate attenuated NKH. In mild attenuated NKH, 53% of patients had onset beyond the first week of life, 7 patients at >4 months of age, which predicted good outcome. There was no significant gender difference in age of onset, DQ, or number of AEDs used, but mean CSF glycine was higher in females than in males (219 vs. 155 μM, $p=0.02$).

Glycine biochemistry. The plasma glycine levels, the CSF glycine levels, and the CSF:plasma glycine ratio were higher in severe NKH than in attenuated NKH (Table 1). Despite substantial overlap in the ranges, the following findings were predictive: a CSF glycine level > 230 μM predicted severe NKH with a sensitivity of 43%, and a CSF:plasma glycine ratio ≤ 0.08 predicted attenuated NKH with a sensitivity of 28%. Within the group of attenuated NKH there was a significant difference in CSF glycine and in the CSF:plasma ratio between subclasses (ANOVA, both $p<0.02$) but not in plasma glycine levels (Fig 1B and C). Attenuated mild NKH had significantly lower CSF glycine levels (good 73 vs. intermediate/poor 121 μM, $p<0.01$) and ratios (good 0.095 vs. intermediate/poor 0.15, $p<0.01$).

In attenuated NKH patients, the continuous outcome variable of DQ correlated significantly with age of onset, number of antiepileptic drugs, CSF glycine level, ratio CSF:plasma glycine, and the glycine index (Table 2 and Fig 2). Multiple linear regression showed a best model of outcome in DQ when related to both age of onset 1.06±0·42 and CSF glycine level -0.19±0.075 µM with constant 53±9.1, p=0.01. Multiple logistic regression identified only two independent predictors for disease severity: age of onset (point estimate 1.526, 95% confidence limits 1.183-1.968, p = 0.001) and CSF glycine levels (point estimate 0.993, 95% confidence limits 0.989-0.997, p < 0.001).

Protein, Enzyme activity, Glycine index. Patients affected with *AMT* mutations (15%) are less common than patients with *GLDC* mutations (85%), with no *GCSH* mutations identified. The proportion of T-protein deficient patients was not significantly different in severe NKH (12/56) versus attenuated NKH (5/42) (chi-square p=0.25). Also, plasma or CSF glycine levels, CSF:plasma glycine ratio, age of onset, and DQ did not differ significantly between P- and T-protein deficient patients. The enzyme activity in liver depended on the affected protein (P-protein 0-10% n=4, T-protein 10-31% n=4), as previously reported.[27] Residual activity in EBV-transformed lymphoblasts (n=10) ranged from 0 to 83% of normal activity, and, as previously noted, proved unreliable with a patient with a severe outcome who had 83% residual activity.[21]

In contrast, the glycine index had a strong relation with outcome (Fig 1D). It was highest in the severely affected patients (average 4.01, range 2.84-4.87, n=8), between 2 and 3 in poor attenuated patients (average 2.69, range 2.30 – 3.10, n=4), and < 2 in all intermediate or mild attenuated patients (average 1.27, range 0.24 – 1.90, n=8) (ANOVA p<0.001). As the glycine index reflects residual glycine catabolism on a whole body level, this indicated that patients with intermediate and mild attenuated NKH had more residual activity than patients with poor attenuated NKH and severe NKH, suggesting a contributing genetic factor.

Genetic studies. The mutation spectrum showed extensive intragenic molecular heterogeneity in classic NKH, including 78 novel mutations in *GLDC* and 18 novel mutations in *AMT.* All mutations identified are listed in Supplementary Table at the cDNA and protein level and were submitted to the Leiden Open Variant Databases (http://databases.lovd.nl/shared/genes) of *GLDC* and *AMT* (Fig 3 and 4). Of the 209 alleles in *GLDC* there were 50.2% missense mutations, 8.1% nonsense mutations, 5.7% frameshift mutations, 13.4% splice site mutations, 20.6% exonic deletions, one exonic duplication, and one in-frame 3 bp deletion resulting in a single amino acid deletion. Of the 114 mutations, 72 mutations were only identified in a single allele. Recurring mutations (≥5 times) included p.R515S (13x), del exon1-2 (12x), IVS22+1G>C (10x), p.A389V (8x), del exon3-21 (7x), p.A802V (6x), p.A202V (5x), p.E167X (5x), and IVS19-1G>A (5x). The exonic deletions were heterogeneous ranging from a single exon to entire gene deletions, and were as frequent on the maternal (21) as the paternal (22) allele. Some patients had two small non-overlapping deletions not detectable by sequencing, making deletion analysis diagnostically imperative. A mutation was not identified in two alleles, giving current analysis methods 99% sensitivity in *GLDC*. The *AMT* gene with 38 alleles had a different mutation spectrum with 76.3% missense mutations, 7.9% splice site mutations, 13.2% frameshift mutations, and one nonsense mutation, but no exonic deletions or duplications. All recurring mutations were missense: p.R320H (7x), p.M1T (4x) and p.I106T (4x). *In silico* analysis confirmed the expected pathogenicity of mutations of each consensus splice site two nucleotides (score < 0.46), except for IVS18-2A>G in *GLDC* (score 0.64), where RNA analysis confirmed the presence of normally spliced mRNA indicating a leaky splicing mutation (data not shown). The *in silico* prediction of pathogenicity of the deep splice site mutation IVS6+5G>C in *AMT* was confirmed by RNA analysis showing the presence of sequence compatible with the insertion of intron 6 sequence. The *in silico* predicted pathogenicity of the deep splice site mutation IVS23+5G>A in *GLDC* could not be further verified as lymphoblasts were unavailable. An ethnic concentration was present for

AMT p.I106T in the Southern Netherlands and *GLDC* p.S132L in New Zealand, reflecting a founder effect. Most parents were carriers, but a *de novo* mutation not present in the blood of the parents was identified in three patients (1% of alleles), although germline mosaicism could not be excluded.

Residual activity of mutations in expression analysis. In many metabolic conditions, residual enzyme activity determines clinical outcome. We analyzed the impact of mutations on residual activity and in relation to neurodevelopmental outcome. There were differences in the mutation classes between severe NKH and attenuated NKH. Nonsense mutations, frameshift mutations, exonic deletions and duplications, which have no residual activity, were more frequent in severe than in attenuated NKH patients (38 % vs 11% of alleles, $p<0.001$), and adding mutations affecting consensus donor and acceptor splice sites, which usually result in absent activity, accentuated this difference (severe NKH 57/109, attenuated NKH 18/84 alleles, $p<0.001$). Patients with both alleles from these classes had severe NKH, whereas each attenuated NKH patient had at least one missense mutation. Two exceptional mutations were classified as mild: a frameshift mutation which affects only the penultimate 20 amino acids, and a leaky splice site mutation IVS18-2A>G.

We hypothesized that missense mutations that recurred in attenuated NKH (p.A202V, p.T269M, p.A389V, p.A802V in *GLDC* and p.I106T in *AMT*), conferred residual enzyme activity, whereas others that occurred in homozygosity in severe NKH patients (p.S132L and p.R515S in *GLDC* and p.R320H and p.M1T in *AMT*), had no residual activity. We expressed selected missense mutations and measured residual activity of the P-protein using the glycine exchange reaction with recombinant human H-protein, which after the lipoyl-ligase reaction was > 80% lipoylated by mass spectrometry, and was no longer rate limiting at 4 μM. The mutations p.S132L, p.P304L, p.C382Y, p.T437I, p.R515S, p.A569T, p.G618R, p.S657R, p.G761R, p.S808R, p.A833V, p.P893L, and p.H950R had no measurable residual activity.

Mutations with residual activity were classified as very mild with >10% activity (p.L82S, p.A202V, p.I381T, p.A389V, p.L548V, p.A802V, p.A802E), mild with activity 1 – 10% (p.T269M, p.C291Y, p.Q366R, p.G607S, p.R630P), and intermediate with measurable activity 0.5 – 1% of wild type activity (p.A283P, p.R461Q, p.A678del) (Supplementary Table).

To relate residual activity to outcome, we analyzed 60 patients who survived the neonatal period with known neurodevelopment outcome, and for whom both mutations in *GLDC* were identified and the residual activity determined (Fig 5). Both developmental outcome as DQ and number of AEDs used differed significantly with the number of alleles with residual activity (Fisher's exact test both $p<0.001$). Patients with 2 severe mutations used multiple (2-4) antiepileptic drugs; patients with 2 residual activity conferring alleles used no or rarely one antiepileptic drug; whereas patients with a single residual activity conferring allele used no (in 47%) or 1-3 antiepileptic drugs. All 29 patients with 2 severe mutations had severe NKH; all 8 patients with 2 mutations with measurable residual activity had attenuated NKH, 6 of them mild, whereas 91% of patients with a single allele with residual activity had attenuated NKH the majority poor or intermediate. Even patients with a single intermediate mutation still had attenuated outcome (patients 18, 61, 79), indicating that very little *in vitro* residual activity is required. Exceptions exist where severe outcome occurred even in the presence of a single very mild allele (e.g. p.A802V (patient 39) and p.A389V (patient 35)). This indicates that an allele with residual activity is necessary but not sufficient for attenuated outcome, and that factors in addition to genotype influence outcome. Further illustrating this, the average DQ was higher in patients with two residual activity conferring mutations than in those with a single such mutation (53 vs 28, $p=0.01$), but within the group of attenuated patients there was no correlation between DQ and the summed residual activity of both alleles ($r^2<0.001$). Substantial phenotypic differences existed within the same genotype; for

instance p.A389V/p.R515S was seen in a severe (patient 35) and an attenuated intermediate phenotype (patient 148).

Expanding this analysis to all patients showed a significant difference in the mutation score between outcome categories (ANOVA $p<0.001$, post hoc Tukey difference only significant between severe 1.38±1.41 and attenuated -1.43±0.83 $p<0.001$, Fig 6). A score of -2 predicted severe outcome with sensitivity of 62% and specificity of 100%, and a score ≥1 predicted attenuated outcome with a sensitivity of 81% and a specificity of 94% (Table 3). Combined, this mutation score had a predictive sensitivity of 70% and specificity of 97%.

Molecular modeling and protein stability. The relative locations of the expressed mutations are shown on the tertiary structure of human P-protein modeled on the homologous $\alpha 2$ homodimer structure of *Synechocystis sp.* glycine decarboxylase (Fig 7).[26] Mutated amino acids located near the active site pocket (A833, L548, H950, A389, S808, I381, Q366, C382, G607, P893) tended to have stable protein levels but highly variable residual activity. For instance, mild mutations Q366R, I381T, and G607S affect amino acids located in a loop or domain that is mobile upon pyridoxal-phosphate binding in *Th. thermophilus*, and Q366 resides in the lipoamide channel.[28] In contrast, mutated amino acids located on the outer side of protein away from the active pocket (A283, A202, C291, T269, R461, P304, R515, S132, A569, A678, T437) tended to have decreased protein levels of variable residual activity, likely due to protein instability. For instance mild mutations p.T269M and p.A202V located in the 9th α-helix, and severe mutations p.R515S and p.G761R affected the structural interaction of the N- and C-terminal halves of the protein.[28] Co-transfection studies were done to review if observed combination of two mutations would affect the residual activity given the dimeric structure of P-protein. The severe but stable mutant p.S657R did not significantly reduce the residual activity or protein levels of p.A802V

(5.9%) (patient 39) compared to sham (7.2%, p=0.28) (patient 52), but the unstable p.R515S decreased the activity and the protein amount of p.A389V (4.2% activity) (patients 35, 148) compared to sham (11.2% p<0·001) (patient 278), indicating a dominant negative effect for unstable mutants at the protein level (Fig 8).

Neonatal and early infantile deceased patients. As a group, neonatally deceased patients were similar to severe patients. They had more severe mutations (65% vs. 54% of alleles, p<0.001), less brain malformations (48% vs. 71%, p<0.001), but similar CSF glycine levels and CSF:plasma glycine ratio. On close observation, we identified a few patients who had residual activity conferring mutations, without brain malformations, and with low CSF glycine level and CSF:plasma glycine ratio (e.g. patients 33a and 33b with p.T269M, and patient 19 with p.G607S). Since >50% of attenuated NKH patients presented neonatally, we presume that these patients at least had the potential for attenuated outcome.

Discussion

At the outset of this study, we encountered patients who presented neonatally and had been given a dismal prognosis, but who made substantial developmental progress. This inaccurate prognostic information was frustrating for the families. Indeed, the majority of patients with attenuated NKH, even mild, presented neonatally, and predicting prognosis based on age at onset alone was inaccurate. Moreover, single case studies of a new treatment, such as dextromethorphan, exhibited the same prognostic pitfall attributing the attenuated outcome to the drug treatment when, because of the neonatal presentation, the patient was expected to have a severe outcome.[29] A more accurate prognostic stratification is required for adequate clinical trial evaluation. A reliable measure to predict outcome in NKH was not yet available,[30] and past small studies had contradictory conclusions on the predictive value of glycine levels.[7,14] In this study, we categorized patients with NKH according to

outcome, rather than age of onset, using developmental attainment as the primary outcome measure, which correlated well with neurological indicators such as the presence of brain malformations and the number of antiepileptic drugs used to treat epilepsy. Attenuated NKH was distinguished from severe NKH by the presence of a variable degree of development,[2] and, despite its continuous nature, was subdivided in categories to facilitate statistical analysis.

This study provides strong evidence that genotype is a primary determinant of outcome, and presence of residual activity relates to outcome, as had previously been hypothesized from individual cases.[7,8,11,22] Two mutations without residual enzyme activity are associated with severe outcome and attenuated outcome requires at least one mutation providing residual enzyme activity. Lack of residual enzyme activity in severe patients results in a larger glycine pool, which requires more sodium benzoate to normalize glycine levels as reflected in a greater glycine index,[18] which showed the best discrimination between outcome categories. This larger glycine pool results in increased brain and CSF glycine levels. CSF glycine correlated significantly though not strongly with outcome measures, but at the extremes CSF glycine > 230 μM predicted severe outcome and low CSF:plasma glycine ratio ≤ 0·08 predicted attenuated outcome. Of the clinically useful predictors, which are listed with sensitivity and specificity in Table 4, age of onset, brain imaging, CSF glycine, and CSF:plasma glycine ratio are available in the immediate newborn period to enable counseling of parents. Using these four predictors available at diagnosis (age of onset ≥4 months, CSF glycine >230 μ, CSF:plasma glycine ratio ≤0.08, and brain malformations on MRI) , 78% of severe patients and 49% of attenuated patients would be accurately identified. A simplified panel of 7 mutations (p.A202V, p.A389V, p.A802V/E, p.T269M, p.L548V, p.G607S in *GLDC* and p.I106T in *AMT*) identified 55% of attenuated patients, and could be implemented for rapid prediction in the neonatal period.

Molecular analysis provides an important predictor. This report greatly expands the number of mutations beyond those previously reported. Combined sequencing and exonic deletion-duplication

analysis identified 99% of causative alleles in *GLDC* and all in *AMT*, and constitutes the preferred confirmatory diagnostic testing. Due to the documented 1% de novo mutation rate, carrier status of parents should be verified and not assumed in the context of recurrence risk counseling and prenatal diagnosis. The large genetic heterogeneity complicates the analysis of genotype to phenotype relation. Caution is necessary when using generic rules in predicting whether a mutation would retain residual activity. Phylogenetic conservation was unhelpful with most mutations affecting conserved amino acids regardless of residual activity, yet p.A569T affects a poorly conserved amino acid but had no residual activity. Mutations affecting the two essential splice donor/acceptor nucleotides severely disrupted splicing, but IVS18-2A>G was a notable exception. To evaluate missense mutations, we developed a new effective expression study with results agreeing with the few previous publications.[27,32] The amount of residual activity associated with attenuated outcome was very low. Mutations that result in unstable protein tend to be located away from the active cleft, and even reduce the protein amount of the other allele likely due to dimer formation as was shown for the p.R515S mutation. In this study, 69/124 patients carried at least one *GLDC* missense mutation, and 13/27 *GLDC* missense mutants created unstable proteins including common mutations such as p.R515S. The impact of current therapy with benzoate and dextromethorphan has been limited. A theoretically new therapeutic option of a chaperone or a proteostasis regulator which increases protein levels could increase enzyme activity and positively affect outcome in at least 27% of patients.[31] The relation between residual activity and outcome is complex. The number of alleles with residual activity relates to outcome, but there was not a direct correlation between DQ and the amount of residual activity in this eukaryotic expression system. Patients with a similar mild mutation sometimes had a very different outcome in this study, and substantial intra-familial variability in large families has been noted.[14] This suggests that factors additional to genotype contribute to outcome.[11,32] For patients with attenuated NKH, the nature and timing of early treatment has been suggested as a one potential factor.[30]

Though this study is the largest ever in NKH, it does have limitations. Patients were evaluated from a very wide geographic region, allowing for generalization in this rare condition, and therefore encountered a variety of methods to evaluate developmental outcome. Analysis of developmental and behavioral outcome subcategories was not possible and will require a systematic analysis at a few centers using standardized measures. A wide spectrum of behavioral and developmental problems was noted in preliminary analysis of a few patients with attenuated NKH. The MRI review was limited to brain malformations as described in the report of the reading radiologist, and detailed analysis, such as the distribution of diffusion restriction, requires further study.[33,34] A detailed description of the seizure type, electroencephalogram, or response to specific antiepileptic medications could not be systematically captured, and requires a separate study. The number of AED used, which could be accurately recorded, is significant since the need for more than two AEDs, as seen in severe NKH, relates to presence of therapy-refractory epilepsy.[35]

This study documented several factors predicting prognosis, including the presence of brain malformations, the CSF glycine level, the CSF:plasma glycine ratio, and the type of genetic mutation. This information is valuable to parents and clinicians to allow better prediction of outcome. Prediction of severe outcome can help families in considering withdrawal of life-sustaining therapy in the neonatal apneic phase given the current lack of effective therapies in this setting.[15,36] It is also important in the stratification of patients in treatment studies. Different treatment responses may exist between severe and attenuated NKH.[30,36] The relation between residual activity and outcome suggests that novel strategies to improve residual activity such as chaperones may have a beneficial clinical effect and should be researched. The complex relationship between genotype and phenotype indicates that additional factors will need to be identified to make such a strategy fully successful.

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Author contributions:

Conception, design and coordination of the study was by J.V.H. Acquisition and analysis of clinical data was by J.V.H., J.H., C.C., K.B., and G.S., and of laboratory data was by M.S., G.S., H.S., E.S., G. C.-S., V.M., G.M., D.A., J.T., and K.W. Statistical analysis was done by J.V.H. and S.T. The manuscript was written by J.V.H, M.S, and C.C.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Figures:

Figure 1: Differences in antiepileptic drugs, glycine levels, and glycine index by disease category

Legend: A. The differences between severe NKH, poor attenuated NKH, intermediate attenuated NKH, and mild attenuated NKH are shown for the number of antiepileptic drugs (A), the CSF glycine levels (B), the CSF:plasma glycine ratios (C), and the glycine index (D).

Figure 2: Relation between CSF glycine levels and DQ

Legend: There is a direct linear correlation between CSF glycine levels and DQ. The cutoff at 230 µM is shown.

Figure 3: Mutations in *GLDC*

Legend: Missense mutations are shown above the diagram of the exonic structure of the *GLDC* gene (A). Missense mutations with residual activity are shown in green, and missense mutations without residual activity are shown in red, mutations not expressed in yellow. Frameshift mutations (grey) and splice site mutations (orange) are shown below the exonic structure. The length of observed exonic deletions is shown in red and duplications in blue. The frequency of occurrence is provided for recurring mutations.

Figure 4: Mutations in *AMT*

Legend: Missense mutations (yellow) are shown above the diagram of the exonic structure of the *AMT* gene. Frameshift mutations (grey) and splice site mutations (orange) are shown below the exonic structure.

Figure 5: Number of residual activity conferring mutations and disease severity

Legend: A. The frequency of developmental outcome in categories of severe, attenuated poor, attenuated intermediate, attenuated mild, or attenuated not otherwise specified (NOS, without known DQ) is shown in relation to the number of alleles with a mutation conferring no residual activity (severe mutation) or alleles with a mutation conferring residual activity (mild mutation). B. The frequency of patients requiring a number of antiepileptic medications in relation to the number of severe or mild alleles.

Figure 6: Mutation score by disease category

Legend: The mutation score is compared between neonatal deceased, severe NKH, poor attenuated NKH, intermediate attenuated NKH, mild attenuated NKH, and attenuated NKH NOS. The mutation score is significantly higher in attenuated NKH than in severe or neonatal decreased NKH.

Figure 7: Molecular modeling of mutated amino acids on the structure of the P-protein

Homology structure of the human P-protein dimer modeled from *Synechocystis sp.* is shown with the amino acids of the expressed missense mutations highlighted. Amino acids involved in missense mutations without residual activity on expression study are shown in red, with < 1% residual activity in blue, with 1-10% residual activity in yellow, and > 10% residual activity in green. The active site lysine 754 is shown in pink. Amino acids which when mutated result in a stable protein (identified in the lower part) tend to cluster around the active site fold (red circle) whereas amino acids which when mutated result in unstable protein (identified in the upper part) tend to be located away from the active site fold.

Figure 8: Western blot analysis of expressed mutations in *GLDC*

Legend: The missense mutations present in two patients were expressed in COS cells, and the level of P-protein identified by Western blot. The amount of β-actin protein is shown as a loading control.

Tables

Table 1: Clinical predictors: Age of onset, Brain malformations, Glycine levels and ratio

Category	Severe	Attenuated all[c]	Attenuated poor	Attenuated intermediate	Attenuated mild
Age of onset					
N	56	42	6	12	15
1st week	49	20	5	5	7
> 1 week	7	22	1	7	8 (> 4 months in 7)
Oldest	2 months	3 year	2 months	4 months	3 year
Brain Malformation					
N (with brain imaging data)	45	40	6	12	15
Corpus callosum agenesis	9	0	0	0	0
Corpus callosum hypoplasia	18	3	1	0	0
Cerebellar cyst/hydrocephalus	4	0	0	0	0
Brain atrophy	1	0	0	0	0
No malformations	13	34	5	12	15

Glycine levels					
Plasma glycine levels µM					
Median, N	1055 , N=48	827 , N=37[a]	844 , N=6	760 , N=12	829 , N=15
Range	324-2048	342-1590	552-1433	509-1120	470-1196
CSF glycine levels µM					
Median, N	213 , N=46	89 , N=31[b]	138 , N=6	89 , N=11	63 , N=11
Range	40-510	41-230	103-172	51-230	41.4-154
CSF:plasma glycine ratio					
Median, N	0.22 , N=45	0.12 , N=29[b]	0.17 , N=5	0.14 , N=11	0.08 , N=9
Range	0.09-0.45	0.04-0.22	0.11-0.21	0.06-0.22	0.04-0.17

Legend: Mann-Whitney-U test a. $p<0.01$; b. $p< 0.001$; c. the category attenuated all also included patients with attenuated NKH not otherwise specified where the DQ is not known.

Table 2: Relation of developmental quotient as outcome and predictive variables

	N	Pearson R^2	p value	Spearman Rho	p value
Attenuated NKH only					
Age onset	33	0.247	0.003	0.384	0.03
Antiepileptic drugs	33	-0.260	0.002	-0.582	<0.001
CSF glycine	28	-0.315	0.002	-0.664	<0.001
CSF:plasma glycine	24	-0.328	0.003	-0.544	0.005
Glycine index	12	-0.424	0.02	-0.765	0.004
Attenuated and Severe					
Age onset	88	0.311	<0.001	0.433	<0.001
Antiepileptic drugs	56	-0.599	<0.001	-0.827	<0.001
CSF glycine	74	-0.296	<0.001	-0.681	<0.001
CSF:plasma glycine	70	-0.289	<0.001	-0.622	<0.001
Plasma glycine	81	-0.081	0.01	-0.266	0.02
Glycine index	20	0.645	<0.001	-0.904	<0.001
Mutation score	81	-0.479	<0.001	0.674	<0.001

Legend: Correlation between DQ and each of the predictive factors.

Table 3: Presumed mutation score as a predictor

Score	Severe	Attenuated
-2	34	0
≥ 1	2	34
Other	19	10
Total	55	42

Legend: Number of severe or attenuated NKH patients with a specific mutation score.

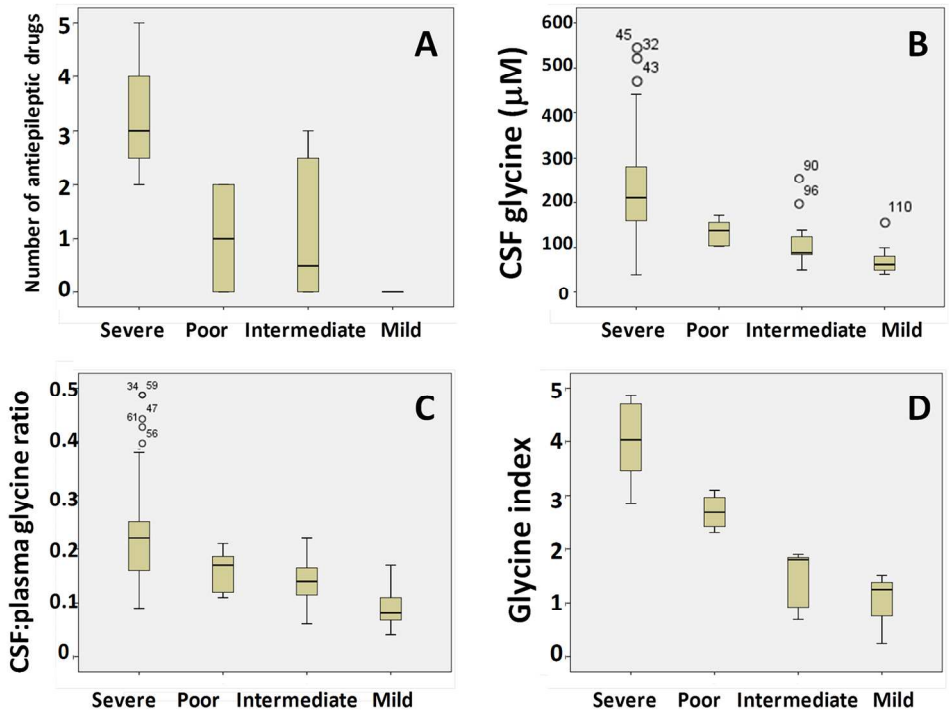
Table 4: Clinically useful predictors of outcome

Parameter	Outcome	Sensitivity	Specificity
Onset > 4 months	Attenuated mild	47%	100%
Onset 1st week	Severe	87%	70%
CSF glycine > 230 µM	Severe	43%	100%
CSF:plasma glycine ratio ≤ 0.08	Attenuated	28%	100%
Brain malformation severe	Severe	29%	100%
Brain malformation all (incl. HCC)	Severe	71%	92%
No epilepsy (no AED)	Attenuated	70%	100%
Glycine index > 3[a]	Severe	87%	100%
2 non-missense mutations	Severe	36%	95%
Mutation score[b] -2	Severe	59%	97%
Mutation score[b] ≥1	Attenuated	76%	94%

Legend: a. A Glycine index of 3 mmol/kg/day calculates for a child on breast milk or regular infant formula with intake of 150 ml/kg/day to a sodium benzoate dose of 510 to 540 mg/kg/day in order to bring glycine levels within therapeutic range (120 – 300 µM). b. The mutation score provides a score of -1 for a mutation expected to leave no residual activity and +2 for a mutation that is expected to have residual activity. The sum of both alleles scored as is currently known is used. HCC = hypoplastic corpus callosum, AED = antiepileptic drugs

Supplementary Table: Clinical and biochemical characteristics in patients with nonketotic hyperglycinemia

Legend: The first tab shows the clinical data, the biochemical data, the genetic results on the maternal and on the paternal allele, and the derived mutation score for each patient sorted by clinical outcome. The second tab shows the combination of expressed mutations in each affected patient. The third tab provides explanations of the abbreviations used in the preceding two tables.



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Legend: A. The differences between severe NKH, poor attenuated NKH, intermediate attenuated NKH, and mild attenuated NKH are shown for the number of antiepileptic drugs (A), the CSF glycine levels (B), the CSF:plasma glycine ratios (C), and the glycine index (D).

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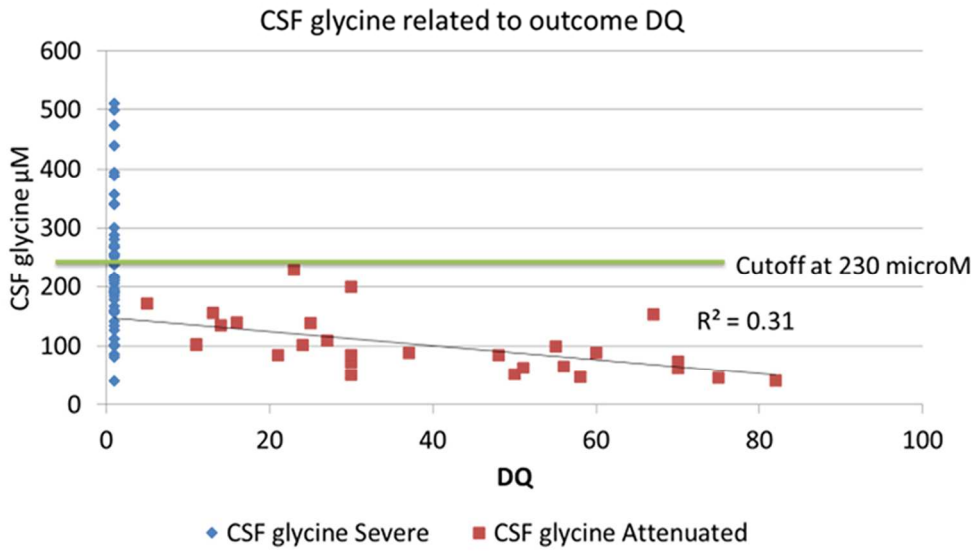

Figure 2: Relation between CSF glycine levels and DQ
Legend: There is a direct linear correlation between CSF glycine levels and DQ. The cutoff at 230 µM is shown.
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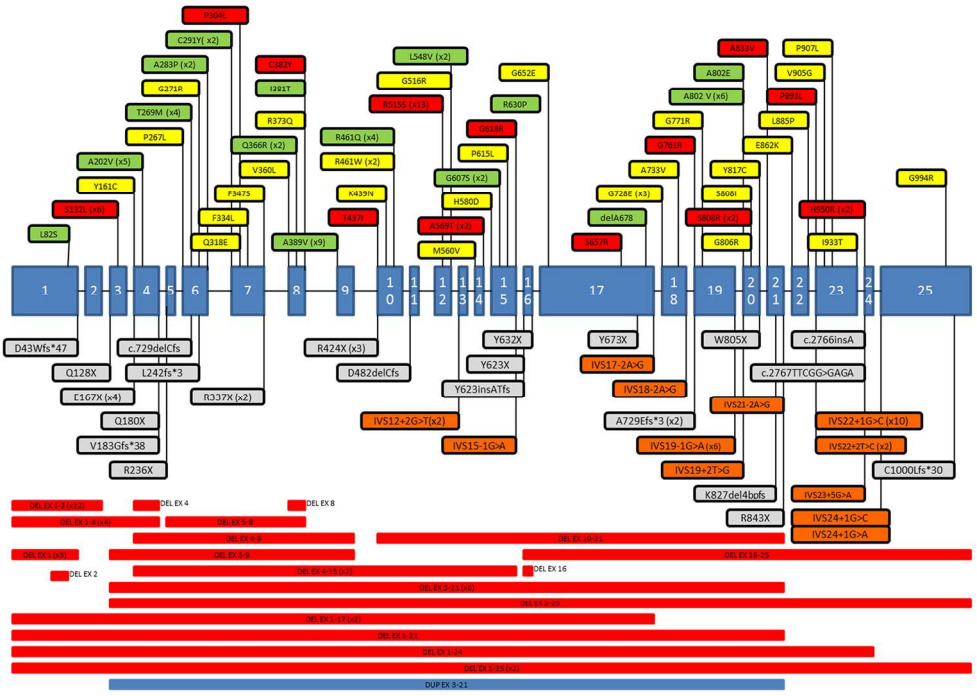

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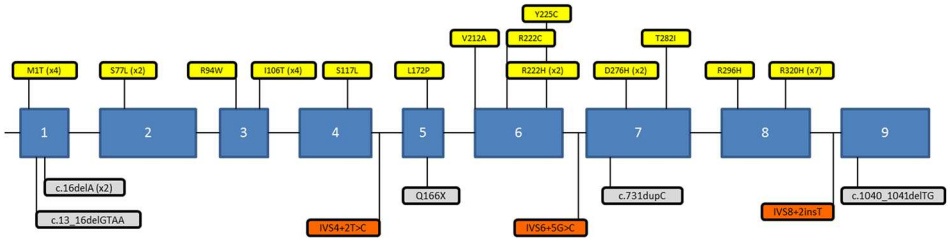

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Legend: Missense mutations (yellow) are shown above the diagram of the exonic structure of the AMT gene. Frameshift mutations (grey) and splice site mutations (orange) are shown below the exonic structure.
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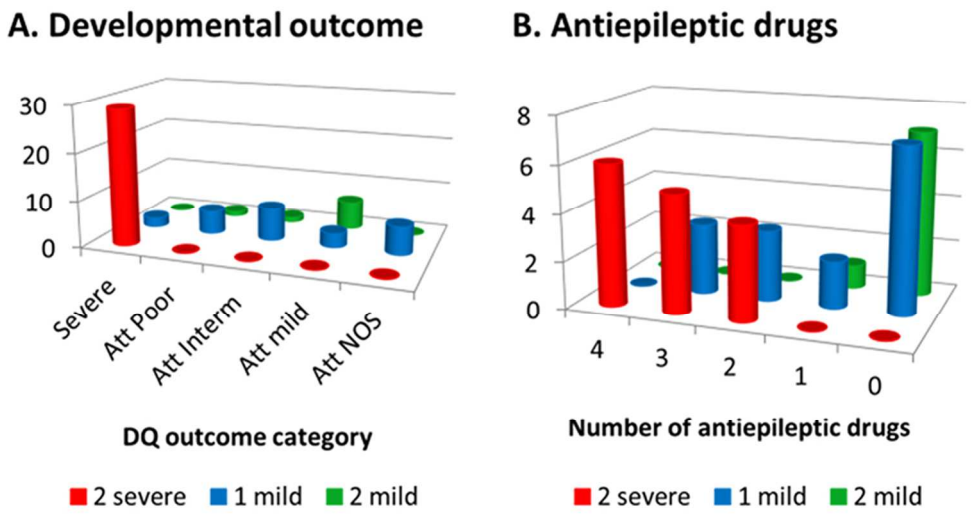

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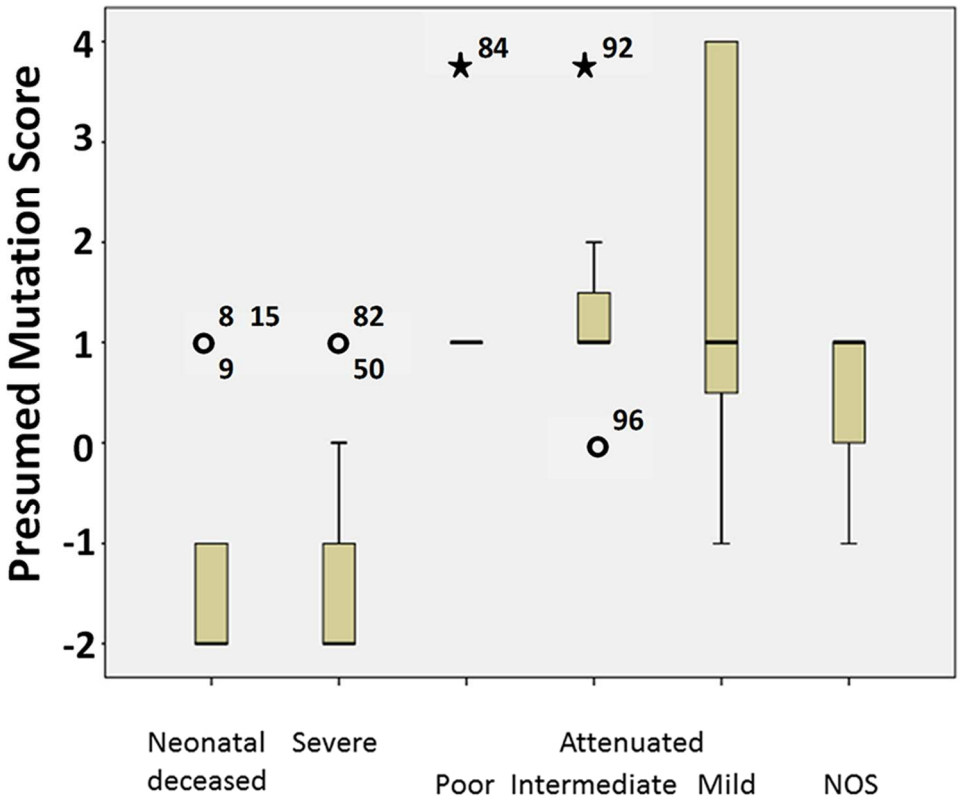

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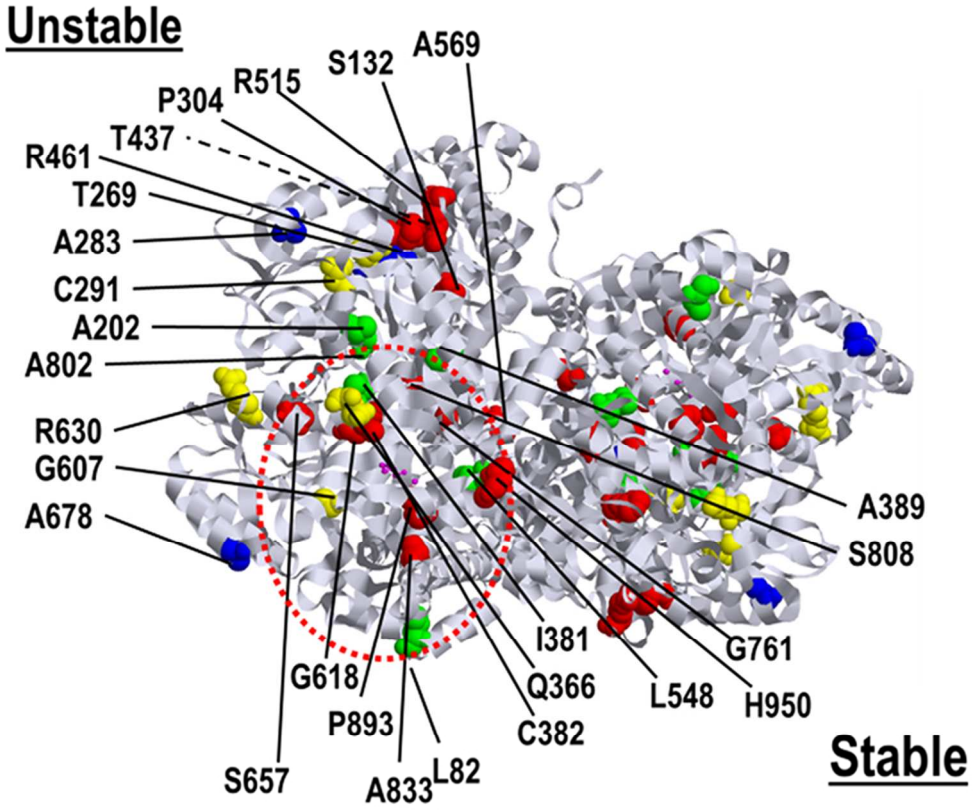

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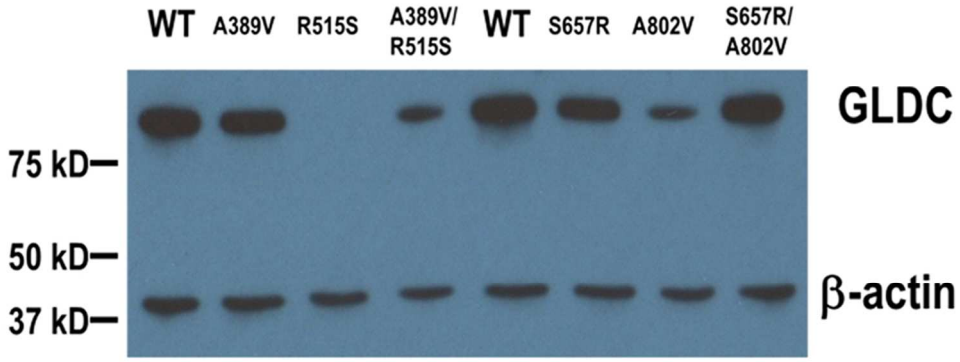

Figure 8: Western blot analysis of expressed mutations in GLDC
Legend: The missense mutations present in two patients were expressed in COS cells, and the level of P-protein identified by Western blot. The amount of β-actin protein is shown as a loading control.
80x31mm (300 x 300 DPI)

Study	Subject	Origin	Category	Gender	Onset	Onset months	Malfor-mations	DQ	AED	Plasma Gly	CSF Gly	Ratio CSF: plasma Gly	Gly Index	Enzyme Activity Liver %	Enzyme Activity Lymp %	Protein	DNA maternal allele	Conserv ation	DNA paternal allele	Conser vation	known mutation score	Presumed mutation score
C	27	USA	ND	M	1 we	0.2	0			1393	294	0.21				P	Del exon 1-2	X	c.2498C>T; p.A833V	7	-2	-2
C	69	BEL	ND	M	1 we	0.2	ACC			940	254	0.27			0	P	c.2422A>C ; p.S808R	3	c.538C>T ; p.Q180X	X	-2	-2
C	66	NET	ND	F	1 we	0.2	ACC			1186	270	0.23				P	c.1009C>T ; p.R337X	X	c.1009C>T ; p.R337X	X	-2	-2
C	227	USA	ND	M	1 we	0.2	ACC			1188	190	0.16				P	Del exon 1-17	X	Del exon 5-8	X	-2	-2
C	168	USA	ND	M	1 we	0.2	ACC			1621	190	0.16				P	c.1545g>c ; p.R515S	7	Del exon 1-2	X	-2	-2
C	249	USA	ND	M	1 we	0.2	0			1546	210	0.14				P	Del exon 3-21	X	Del exon 3-21	X	-2	-2
C	96	USA	ND	F	1 we	0.2	0			1200	176	0.15				P	Del exon 1-2	X	c.1545G>C ; p.R515S	7	-2	-2
C	33a	USA	ND	F	1 we	0.2	0			888	87	0.10				P	c.806C>T ; p.T269M	7	Del exon 1-2	X	1	1
C	33b	USA	ND	F	1 we	0.2	0			808	185	0.23				P	c.806C>T ; p.T269M	7	Del exon 1-2	X	1	1
P	60	USA	ND	F	1 we	0.2	0			810	220	0.27				P	Del exon 1-2	X	Del exon 1-2	X	-2	-2
P	86	USA	ND	F	1 we	0.2	ACC									P	c.706C>T ; p.R236X	X	Del exon 10-21	X	-2	-2
P	6	DEK	ND	F	1 we	0.2	0			1495	350	0.23				P	c.2183G>A ; p.G728E	6	c.1545G>C ; p.R515S	7	-1	-1
P	13	USA	ND	F	1 we	0.2	HCC			832	135	0.16				P	Del exon 1-21	X	c.1317G>T ; p.K439N	4	-1	-1
P	15	USA	ND	M	1 we	0.2	ACC			1533	319	0.21				P	Del exon 1	X	c.1851-1G>A ; IVS15-1G>A	S	-2	-2
P	19	USA	ND	M	1 we	0.2	0			1000	96	0.10				P	c.1819G>A ; p.G607S	7	Del exon 3-9	X	1	1
P	20	USA	ND	F	1 we	0.2	HCC			1700	400	0.28				P	c.1545G>C ; p.R515S	7	c.2665+1G>C ; IVS22+1G>C	S	-2	-2
P	41	USA	ND	F	1 we	0.2	HCC									P	Del exon 1	X	c.2450A>G ; p.Y817C	7	-1	-1
P	44	USA	ND	F	1 we	0.2	0			1200	100	0.15				P	c.2678C>T ; p.P893L	7	c.1545G>C ; p.R515S	7	-2	-2
P	50	USA	ND	F	1 we	0.2	0			763	150	0.19				P	c.1678A>G ; p.M560V	7	c.499G>T ; p.E167X	X	-1	-1
P	97	USA	ND	M	1 we	0.2	0			2363	315	0.13				P	c.2665+1G>C ; IVS22+1G>C	S	c.2527C>T ; p.R843X	X	-2	-2
P	85	USA	ND	F	1 we	0.2	n.a.			1580	237	0.15				P	c.729delCfs ; p.L242Lfs*3	X	c.2665+1G>C ; IVS22+1G>C	S	-2	-2
P	12	USA	ND	F	1 we	0.2	ACC			1212	277	0.23				T	c.344+2+2insT; IVS8+2insT	S	c.230C>T ; p.S77L	7	-1	-1
P	43	USA	ND	M	1 we	0.2	n.a.			836	256	0.31				T	c.16delA ; p.S6Vfs*90	X	c.16delA ; p.S6Vfs*90	X	-2	-2
P	24	USA	ND	M	1 we	0.2	0			939	211	0.22				P	c2186delC ; p.A729Efs*3	X	c2186delC ; p.A729Efs*3	X	-2	-2
P	95	BEL	D2Mo	F	1 we	0.2	n.a.			708	138	0.19				P	c.2422A>C ; p.S808R	3	c.952C>G ; p.Q318E	7	-1	-1
C	42	CAN	D3mo	M	1 we	0.2	ACC			1446	200	0.14			0	P	Dup exon 3-21	X	Del exon 16	X	-2	-2
C	107	NET	Severe	M	1 we	0.2	Thin CC	≤ 6 we	4	539	205	0.38				P	c.2849A>G ; p.H950R	7	c.2849A>G ; p.H950R	7	-2	-2
C	67	BEL	Severe	F	1 we	0.2	CerCyst	≤ 6 we	4	830	218	0.29	4.58		0	P	Del exon 1-2	X	Del exon 4	X	-2	-2
C	68	USA	Severe	M	1 we	0.2	CerCyst	≤ 6 we	3	795	167	0.21	4.87			P	Del exon 3-21	X	n.a.∫			
C	70	BEL	Severe	F	1 we	0.2	HCC	≤ 6 we	3	1013	112	0.11			30	T	c.731dupC; A244Afs*32	X	c.959G>A ; p.R320H	7	-1	-2
C	71	GER	Severe	F	1 we	0.2	HCC	≤ 6 we	3	720	160	0.22				P	c.1869T>G ; p.Y632X	X	c.2281G>C ; p.G761R	7	-2	-2
C	272	USA	Severe	M	1 we	0.2	HCC	≤ 6 we	4	1876	499	0.26	3.30			P	Del exon 1-2	X	Del exon 3-21	X	-2	-2
C	38	USA	Severe	M	1 we	0.2	HCC	≤ 6 we	2	1044	341	0.33				P	Del exon 3-25	X	c. 382C>T; p. Q128X	X	-2	-2
C	240	USA	Severe	F	1 we	0.2	ACC	≤ 6 we	4	594	267	0.45				P	c.1545G>C ; p.R515S	7	c.1545G>C ; p.R515S	7	-2	-2
C	318	UK	Severe	M	1 we	0.2	ACC	≤ 6 we	2	1241	190	0.15				P	c.1545G>C ; p.R515S	7	c.2316-1G>A ; p.IVS19-1G>A	S	-2	-2
C	73	GER	Severe	M	1 we	0.2	0	≤ 6 we	3	424	40	0.09	3.62	23	83	T	c.959G>A ; p.R320H	7	c.665G>A; p.R222H	7	0	-1
C	74	GER	Severe	M	1 we	0.2	0	≤ 6 we	2	830	156	0.19		10		T	c.887G>A ; p.R296H	7	c.845C>T ; p.T282I	7	0	0
C	75	GER	Severe	M	6 we	6	0	≤ 6 we	3	1337	184	0.14	4.81		0	T	c.496C>T ; p.Q166X	X	c.696+5G>C ; IVS6+5G>C‡	S	-2	-2
C	173	POR	Severe	F	1 we	0.2	HCC	≤ 6 we	3	469	112	0.24				P	Del exon 1-2	X	Del exon 1-2	X	-2	-2
C	121	TAI	Severe	M	1 we	0.2	HCC	≤ 6 we	5	1287	267	0.21				T	c.826G>C ; p.D276H	7	c.826G>C ; p.D276H	7	0	0
C	108	USA	Severe	M	2 mo	2	ACC	≤ 6 we	2	706	210	0.30	4.04			T	c.280C>T ; p.R94W	7	c.471+2T>C ; IVS4+2T>C	S	-1	-1
C	207	USA	Severe	F	1 we	0.2	0	≤ 6 we	4	502	127	0.25				P	c.127_128delAfs*47 ; p.D43Wfs*47	X	c.2919+1G>C ; IVS24+1G>C	S	-2	-2
C	312	PUE	Severe	F	1 we	0.2	ACC	≤ 6 we	2	1944	474	0.24				P	c.499G>T ; p.E167X	X	c.499G>T ; p.E167X	X	-2	-2
C	186	USA	Severe	F	1 we	0.2	Hydroc	≤ 6 we	4	1559	340	0.22				P	Del exon 1-2	X	c.2665+1G>C ; IVS22+1G>C	S	-2	-2
C	316	USA	severe	F	1 we	0.2	HCC	≤ 6 we	3	2084	510	0.24	2.84			P	c.2665+2T>C ; IVS22+2T>C	S	Del exon 8	X	-2	-2
C	327	USA	Severe	M	1 we	0.2	0	≤ 6 we	2	1370	395	0.29				P	Del exon 1-17	X	c.2665+1G>C ; IVS22+1G>C	S	-2	-2
C	72	SWE	Severe	M	2 mo	2	0	< 6we	4	342	142	0.42	4.02	0	39	P	unknown		c.1705G>A ; p.A569T	2	-1	-1
C	329	GER	Severe	M	1 we	0.2	HCC	≤ 6 we	3	1080	252	0.23		10		P	c.911C>T ; p.P304L	6	c.1705G>A ; p.A569T	2	-2	-2
C	225	USA	Severe	M	1 we	0.2	ACC	≤ 6 we#	3	1400	243	0.17				P	c.499G>T ; p.E167X	X	c.395C>T ; p.S132L	6	-2	-2
P	39	USA	Severe	F	1 we	0.2	0			1500	300	0.2				P	c.1969A>C; S657R	7	c.2405C>T ; p.A802V	5	1	1
P	4	USA	Severe	M	1 we	0.2	n.a.			491						P	c.1381C>T ; p.R461W	7	c.1381C>T ; p.R461W	7	0	0
P	5	USA	Severe	M	1 we	0.2	0			1385	288	0.21				P	c.2919+1G>A ; IVS24+1G>A	S	c.2250-1G>A ; IVS19-1A>G	S	-2	-2
P	7	USA	Severe	F	1 we	0.2	ACC			1797	255	0.15				P	Del exon 1-24	X	c.2720C>T ; p.P907L	7	-1	-1
P	10	USA	Severe	F	1 we	0.2	HCC			1811	389	0.21				P	c.1545G>C ; p.R515S	7	c.1852G>A ; p.G618R	7	-2	-2
C	11	USA	Severe	F	1 we	0.2	n.a.		2							P	c.2250-1G>A ; IVS19-1A>G	S	c.2198C>T ; p.A733V	6	-1	-1

Study	Subject	Origin	Category	Gender	Onset	Onset months	Malfor-mations	DQ	AED	Plasma Gly	CSF Gly	Ratio CSF: plasma Gly	Gly Index	Enzyme Activity Liver %	Enzyme Activity Lymp %	Protein	DNA maternal allele	Conserv ation	DNA paternal allele	Conser vation	known mutation score	Presumed mutation score
P	14	USA	Severe	F	1 we	0.2	HCC			1140	440	0.39				P	c.1270C>T ; p.R424X	X	c.1270C>T ; p.R424X	X	-2	-2
P	21	NWZ	Severe	F	1 we	0.2	0			1117	257	0.23				P	c.495C>T ; p.S132L	6	c.495C>T ; p.S132L	6	-2	-2
P	23	NWZ	Severe	M	n.a.		n.a.									P	c.495C>T ; p.S132L	6	c.2423G>T ; p.S808I	3	-1	-1
P	25	USA	Severe	F	1 we	0.2	HCC			438	197	0.45				P	c.2481_2484delACAA ; p.K827Kfs*7	X	c.2416G>C ; p.G806R	7	-1	-1
P	28	USA	Severe	F	1 we	0.2	0			1264	189	0.15				P	c.1444delG ; p.D482Tfs*57	X	c.2250-1G>A ; IVS19-1A>G	S	-2	-2
P	30	USA	Severe	F	1 we	0.2	0			582	238	0.41				P	Del exon 1-4	X	Del exon 1-4	X	-2	-2
P	34	USA	Severe	M	1 we	0.2	n.a.									P	c.2980G>A ; p.G994R	7	c.2584G>A ; p.E862K	7	0	0
P	46	USA	Severe	M	1 we	0.2	ACC			869	191	0.22				P	c.2250-1G>A ; IVS19-1A>G	S	c.1545G>C ; p.R515S	7	-2	-2
P	48	USA	Severe	F	1 we	0.2	n.a.									P	c.800C>T ; p.P267L	6	2415G>A ; p.W805X	X	-1	-1
P	53	USA	Severe	F	1 we	0.2	HCC			1626	281	0.17				P	c.2458-2A>G ; IVS21-2A>G	S	Del exon 4-9	X	-2	-2
P	54	USA	Severe	F	1 we	0.2	HCC			1326	178					P	c.2021T>A ; p.Y673X	X	c.2654T>C ; p.L885P	7	-1	-1
P	58	USA	Severe	M	2 we	0.5	HCC			990	215	0.22				P	c.2250-1G>A ; IVS19-1A>G	S	c.2311G>A ; p.G771R	6	-1	-1
P	62	NWZ	Severe	F	1 we	0.2	n.a.			1354	271	0.2				P	c.1310C>T ; p.T437I	7	c.495C>T ; p.S132L	6	-2	-2
P	82	USA	Severe	F	1 we	0.2	HCC, SG					0.16				P	Del exon 1-25	X	c.1000T>C ; p.F334L	5	-1	-1
P	83	USA	Severe	M	1 we	0.2	ACC				100					P	Del exon 1-4	X	Del exon 1-4	X	-2	-2
P	87	USA	Severe	F	2 we	0.5	n.a.			900						P	c.2665+2T>C ; IVS22+2T>C	S	c.1869insAT ; p.Y623Mfs*10	X	-2	-2
P	98a	USA	Severe	M	1 we	0.4	ACC			1816						P	p.I926Nfs*6	X	Del exon 4-15	X	-2	-2
P	98b	USA	Severe	F	1 we	0.4	ACC			1537	357	0.23				P	p.I926Nfs*6	X	Del exon 4-15	X	-2	-2
P	99	USA	Severe	M	1 we	0.4	n.a.			1914	211	0.11				P	c.2665+1G>C ; IVS22+1G>C	S	unknown	n.a.	-1	-1
P	2	USA	Severe	M	1 mo	1	0			610	87	0.14				T	c.2T>C; p.M1T	5	c.2T>C; p.M1T	5	0	-2
P	17	GRE	Severe	M	1 we	0.2	HCC			573	82	0.14				T	c13_16delGTAA ; p.S5Vfs*27	X	c.350C>T ; p.S117L	6	-1	-1
P	22	USA	Severe	F	1 we	0.2	n.a.			610	134	0.22				T	c.2T>C; p.M1T	5	c.2T>C; p.M1T	5	0	-2
P	47	USA	Severe	F	6 we	1.5	HCC, DW			714	103	0.14				T	c.674G>C ; p.Y225C*	7	c.635T>C ; p.V212A	7	0	0
P	56	USA	Severe	F	1 we	0.2	n.a.									T	c.959G>A ; p.R320H	7	c.959G>A ; p.R320H	7	0	-2
P	59	USA	Severe	M	1 we	0.2	BA									T	c.1040_1041delTG ; p.V347Dfs*2	X	c.230C>T ; p.S77L	7	-1	-1
C	204	USA	Severe	M	1 we	0.2	n.a.	3		1067	193	0.18				P	c.1869T>G ; p.Y623X	X	Del exon 1	X	-2	-2
C	35	USA	Severe	F	1 we	0.2	HY			969	160	0.16				P	c.1166C>T ; p.A389V	7	c.1545G>C ; p.R515S	7	1	1
C	330	GER	Att poor	M	1 we	0.2	0	5	1	925	172	0.19				P	c.1642C>G ; p.L548V*	7	Del exon 1-25	X	1	1
C	76	ITA	Att Poor	M	1 we	0.2	0	11	0	867	104	0.12	2.83			P	c.1097A>G ; p.Q366R	7	c.1097A>G ; p.Q366R	7	4	4
C	245a	USA	Att Poor	M	1.5 mo	1.5	0	11	0	552	103		2.30			P	c.2405C>T ; p.A802V	5	c.2665+1G>C ; IVS22+1G>C	S	1	1
C	310	USA	Att Poor	M	1 we	0.2	HCC	12	1	820	140	0.17	3.10			P	c.2203-2A>G ; IVS18-2A>G	S	c.547delGfs*38 ; p.V183Gfs*38	X	-1	1
C	165	USA	Att Poor	F	1 we	0.2	0	13	2	1433	156	0.11				P	c.2405C>T ; p.A802V	7	c.1545 G>C ; p.R515S	7	1	1
C	45a	USA	Att Poor	F	1 we	0.2	0	14	2	637	136	0.21	2.52			P	c.1580+2T>G ; p.IVS12+2T>G	S	c.1166C>T ; p.A389V	7	1	1
C	78	NET	Att Intermediate	M	1 we	0.2	0	21	1	675	85	0.13	1.90	17	53	T	c.959G>A ; p.R320H	5	c.317T>C ; p.I106T	7	0	1
C	77	NET	Att Intermediate	F	1 we	0.2	0	23	0	1068	230	0.22	0.92	31	39	T	c.317T>C ; p.I106T	7	c.515T>C ; p.L172P	7	0	2
C	148	USA	Att Intermediate	M	3 mo	3	0	24	3	606	103	0.17				P	c.1166C>T p. A389V†	7	c.1545G>C p.R515S†	7	1	1
C	248	USA	Att Intermediate	F	6 we	6	0	25	1	859	139	0.16				P	c.1142T>C ; p.I381T	7	c.1382G>A ; p.R461Q	7	4	4
C	61a	USA	Att Intermediate	M	1 we	0.2	0	27	3	1001	110	0.11				P	c.847G>C ; p.A283P	5	c.1382G>A ; p.R461Q	6	1	1
C	61b	USA	Att Intermediate	M	3 mo	3	0	30	2	509	73	0.14				P	c.847G>C ; p.A283P	5	c.1382G>A ; p.R461Q	6	1	1
C	45b	USA	Att Intermediate	F	3 mo	3	0	30	3	567			0.68			P	c.1580+2T>G ; p.IVS12+2T>G	S	c.1166C>T ; p.A389V	7	1	1
C	79	GER	Att Intermediate	M	1 we	0.2	0	30	0	1120	200	0.18	1.80		30	P	c.1382G>A ; p.R461Q	6	c.1929-2A>G ; IVS17-2A>G	S	1	1
C	159	NET	Att Intermediate	F	2 mo	2	0	30	0	826	85	0.10				T	c.317T>C ; p.I106T	7	c.665G>C ; p.R222H	7	0	2
C	234	YEM	Att Intermediate	M	4 mo	4	0	30	0	850	51	0.06				P	c.482A>G ; p.Y161C	7	c.1041A>C ; p.R347S	7	0	0
C	32a	CAN	Att Intermediate	F	6 we	1.5	0	37	0	578	89	0.15				P	c.605C>T ; p.A202V	7	c.2665+1G>C ; IVS22+1G>C	S	1	1
C	245b	USA	Att Intermediate	M	1 we	0.2	0	48	0	693	85	0.12	1.84			P	c.2405C>T ; p.A802V	5	c.2665+1G>C ; IVS22+1G>C	S	1	1
C	40	NET	Att Good	M	5 mo	5	0	50	0	470	53	0.11				T	c.317T>C ; p.I106T	7	c.959G>A ; p.R320H	7	0	1
C	81a	USA	Att Good	M	1 we	0.2	0	51	0	920	63	0.07			24	P	c.1166C>T ; p.A389V	7	c.1166C>T ; p.A389V	7	4	4
C	81b	USA	Att Good	F	1 we	0.2	0	52	0	915					10	P	c.1166C>T ; p.A389V	7	c.1166C>T ; p.A389V	7	4	4
C	80	GER	Att Good	F	12 mo	12	0	55	0	890	100	0.11	1.51		0	P	c.2999delG fs*30 ; p.C1000Lfs*30	X¶	c.1270C>T ; p.R424X	X	-1	1
C	322	DK	Att Good	M	11 mo	11	0	56	0	829	66	0.08				P	c.466G>T ; p.G516R	5	c.2183G>A ; p.G728E	6	0	0
C	18	USA	Att Good	M	1 we	0.2	0	58	0	1196						P	Del exon 3-21	X	c.2032-2034delCAG ; p.A678del	7	1	1
C	32b	CAN	Att Good	M	1 we	0.2	0	58	0	720	48	0.07				P	c.605C>T ; p.A202V	7	c.2665+1G>C ; IVS22+1G>C	S	1	1
C	298	BEL	Att Good	M	1 we	0.2	0	60	0	584	88.7	0.15	0.24			P	c.1889G>C ; p.R630P	7	c.1642C>G ; p.L548V	7	4	4
C	16	USA	Att Good	M	3 yr	36	0	60	0	697						T	c.959G>A ; p.R320H	7	c.664C>T ; p.R222C	7	0	-1
C	222	USA	Att Good	F	1 we	0.2	0	67	0	930	154	0.17	1.25			P	c.2315+2T>G ; IVS19+2T>G	S	c.2405C>A ; p.A802E	5	1	1

Study	Subject	Origin	Category	Gender	Onset	Onset months	Malfor-mations	DQ	AED	Plasma Gly	CSF Gly	Ratio CSF: plasma Gly	Gly Index	Enzyme Activity Liver %	Enzyme Activity Lymp %	Protein	DNA maternal allele	Conserv ation	DNA paternal allele	Conser vation	known mutation score	Presumed mutation score
C	196	USA	Att Good	M	4 mo	4	0	70	0	800						P	c.806C>T ; p.T269M	7	c.806C>T ; p.T269M	7	4	4
C	218	USA	Att Good	M	10 mo	10	0	70	0	645	74					P	c.2714T>C ; p.V905G	2	c.2183G>A ; p.G728E	6	0	0
C	92	USA	Att Good	F	2 yr 4 mo	28	0	70	0	930	62					P	c.1955G>A ; p.G652E	7	c.1118G>A ; p.R373Q*	6	0	0
C	51	AUS	Att Good	M	1 we	0.2	0	75	0	713	46	0.06				P	c.872G>A ; p.C291Y	2	c.872G>A ; p.C291Y	2	4	4
P	315	CAN	Att Good	M	3 yr	36	0	82	0	932	41.4	0.04				P	c.605C>T ; p.A202V	7	c.605C>T ; p.A202V	7	4	4
P	52	USA	Attenuated NOS	M	7 we	1.75	0			1590	122	0.077				P	Del exon 3-21	X	c.2405C>T ; p.A802V	5	1	1
P	294	NOR	Attenuated NOS	F	2 we	0.5	0			503	91	0.18				P	c.1145G>A ; p.C382Y	7	c.2405C>T ; p.A802V	5	1	1
P	31	NWZ	Attenuated NOS	F	3 mo	3	0			342	43	0.12				P	c.495C>T ; p.S132L	6	c.245T>C ; p.L82S	3	1	1
P	55	USA	Attenuated NOS	M	7 mo	7	n.a.			1011	50	0.05				P	c.1078G>C ; p.V360L	2	c.2838G>A ; IVS23+5G>A	S	-1	-1
P	90	USA	Attenuated NOS	M	1 we	0.2	HCC			758	67	0.08				P	c.2798T>C ; p.I933T	4	c.811G>A ; p.G271R	7	0	0
P	9	USA	Attenuated NOS	M	1 mo	1	n.a.									P	c.1819G>A ; G607S	7	c1580delGinsCAA ; p.S527Tfs*13	X	1	1
P	296	USA	Attenuated NOS	M	1 we	0.2	HCC			1284						P	c.1738C>G ; p.H580D	7	c.1844C>T ; p.P615L	7	0	0
P	325	NWZ	Attenuated NOS	F	6 we	1.5	0			590	55	0.09				P	Del exon 16-25	X	c.605C>T ; p.A202V	7	1	1
P	278	USA	Attenuated NOS	M	1 we	0.2	0			870	175	0.21				P	c.1166C>T ; p.A389V	7	Del exon 2	X	1	1

cDNA	Protein	Western Blot	Activity	Effect	Subject	Other allele	Clinical severity
c.245T>C	p.L82S	Stable	34 % ± 8.9	Very mild	31	p.S132L severe	Attenuated
c.495C>T	p.S132L	Unstable	BDL	Severe	225	p.E167X severe	Severe
					21	Homozygous	Severe
					23	p.S808I likely severe	Severe
					62	p.T437I severe	Severe
					31	p.L82S very mild	Attenuated
c.605C>T	p.A202V	Less Stable	18.2 % ± 3.43	Very mild	325	Del Ex 16-25	Attenuated
					32a	IVS22+1G>C severe	Attenuated intermediate
					32b	IVS22+1G>C severe	Attenuated mild
					315	Homozygous	Attenuated mild
c.806C>T	p.T269M	Unstable	1.3 % ± 0.21	Mild	196	Homozygous	Attenuated mild
					33a	Deletion exon 1-2 severe	Neonatal death, no poor sign
					33b	Deletion exon 1-2 severe	Neonatal death, no poor sign
c.847G>C	p.A283P	Unstable	0.5%± 0.043	Intermediate	61a	p.R461Q	Attenuated intermediate
					61b	p.R461Q	Attenuated intermediate
c.873C>G	p.C291Y	Unstable	1.1 % ± 0.33	Mild	51	Homozygous	Attenuated mild
c.911C>T	p.P304L	Unstable	BDL	Severe	329	p.A569T severe	Severe
c.1097A>G	p.Q366R	Stable	1.1 % ± 0.19	Mild	76	Homozygous	Attenuated poor
c.1142C>T	p.I381T	Stable	32.3 % ± 8.69	Very mild	248	p.R461Q Intermediate	Attenuated intermediate
c.1145G>A	p.C382Y	Stable	BDL	Severe	294	p.A802V Very mild	Attenuated
c.1166C>T	p.A389V	Stable	11.9 % ± 2.53	Very mild	35	p.R515S severe	Severe
					45a	IVS12+2T>G severe	Attenuated poor
					148	p.R515S severe	Attenuated intermediate
					45b	IVS12+2T>G severe	Attenuated intermediate
					81a	Homozygous	Attenuated mild
					81b	Homozygous	Attenuated mild
					278	Deletion exon 2	Attenuated
c.1310C>T	p.T437I	Unstable	BDL	Severe	62	p.S132L severe	Severe
c.1382G>A	p.R461Q	Less Stable	0.65% ± 0.27	Intermediate	248	p.I381T very mild	Attenuated intermediate
					61a	p.A283P intermediate	Attenuated intermediate
					61b	p.A283P intermediate	Attenuated intermediate
					79	IVS17-2A>G severe	Attenuated intermediate
c.1545G>C	p.R515S	Unstable	BDL	Severe	240	Homozygous	Severe
					318	IVS19-1G>A severe	Severe

					10	p.G618R severe	Severe
					46	IVS19-1G>A severe	Severe
					168	Deletion exon 1-2	Neonatal death, ACC
					6	p.G728E unknown	Neonatal death, CSF gly 350
					20	IVS22+1G>C severe	Neonatal death, HCC CSF gly 400
					44	p.P893L severe	Neonatal death, no indicator
					96	Deletion exon 1-2 severe	Neonatal death, no indicator
					35	p.A389V very mild	Severe
					148	p.A389V very mild	Attenuated intermediate
					165	p.A802V very mild	Attenuated poor
c.1642C>G	p.L548V	Stable	22.5% ± 3.08	Very mild	330	Deletion exon 1-25	Attenuated poor
					298	p.R630P mild	Attenuated mild
c.1705G>A	p.A569T	Unstable	BDL	Severe	329	p.P304L severe	Severe
					72	Unknown	Severe
c.1819G>A	p.G607S	Unstable	2.04% ± 0.57	Mild	9	p.S527Tfs*13 severe	Attenuated
					19	Deletion exon 3-9 severe	Neonatal death, no indicator
c.1852G>A	p.G618R	Stable	BDL	Severe	10	p.R515S severe	Severe
c.1889G>C	p.R630P	Less Stable	10% ± 1.0	Mild	298	p.L548V very mild	Attenuated mild
c.1969A>C	p.S657R	Stable	BDL	Severe	39	p.A802V very mild	Severe
c.2032delCAG	p.A678del	Less Stable	0.30% ± 0.12	Intermediate	18	Deletion exon 3-21	Attenuated mild
c.2281G>C	p.G761R	Stable	BDL	Severe	71	p.Y632X severe	Severe
c.2405C>T	p.A802V	Less Stable	25.8 % ± 4.4	Very mild	52	Deletion ex 3-21	Attenuated
					245a	IVS22+1G>C severe	Attenuated poor
					245b	IVS22+1G>C severe	Attenuated intermediate
					165	p.R515S severe	Attenuated poor
					294	p.C382Y severe	Attenuated
					39	p.S657R severe	Severe
c.2405C>A	p.A802E	Stable	8.8 % ± 0.73	Mild	222	IVS19+2T>G severe	Attenuated mild
c.2422A>C	p.S808R	Stable	BDL	Severe	69	p.Q180X severe	Neonatal death, ACC
					95	p.Q318E unknown	Infantile death, no indicator
c.2498C>T	p.A833V	Stable	BDL	Severe	27	Deletion exon 1-2	Neonatal death
c.2678C>T	p.P893L	Stable	BDL	Severe	44	p.R515S severe	Neonatal death, no indicator
x.2849A>G	p.H950R	Stable	BDL	Severe	107	Homozygous	Severe

ACC, agenesis of the corpus callosum
BA, brain atrophy
DW, Dandy Walker
HCC, hypoplastic corpus callosum
HY, hydrocephalus
NA, not available
SC, simplified gyral pattern
a and b are sibling pairs
* de novo mutation
# can roll from back to prone
‡ proven to affect splicing
¶ near end of protein, results in residual activity
† phase not known
AED: number of antiepileptic drugs
Study: C= primary study with consent and complete data;
Study: P = secondary exempt chart review study with limited data available
Gly = glycine
Enzyme activities are listed as % of normal control
Gly Index = glycine index, molar difference of glycine intake and benzoate needed to normalize plasma glycine

Conservation: X = not a missense mutation, a number = number of species in which the mutated amino acid is conserved from the 7 species listed in the methods (including human)
For GLDC: Mus musculus, Caenorabdhitis elegans, Drosophila, melanogaster, Saccharomyces cerevisiae, Eschiria coli and Arabidopsis thaliana
For AMT: Pan troglodytes, Bos taurus, Danio rerio, Saccharoyces cerevisiae, and Caenorabdhitis elegans
ʃ only maternal sample available